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Cell Kinetics of GM-CFC in the Steady State

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Abstract. The kinetics of cell turnover for myeloid/monocyte cells that form colonies in agar (GM-CFC) were measured through the progressive increase in their sensitivity to 313-nm light during a period of cell labeling with BrdCyd. Two components of cell killing with distinctly separate labeling kinetics revealed both the presence of two generations within the GM-CFC compartment and the properties of the kinetics of the precursors of the GM-CFC. These precursors of the GM-CFC were not assayable in a routine GM-CFC assay when pregnant mouse uterus extract and mouse L-cell-conditioned medium were used to stimulate colony formation but were revealed by the labeling kinetics of the assayable GM-CFC. Further, these precursor cells appeared to enter the assayable GM-CFC population from a noncycling state. This was evidenced by the failure of the majority of these cells to incorporate BrdCyd during five days of infusion. The half-time for cell turnover within this precursor compartment was measured to be approximately 5.5 days. Further, these normally noncycling cells proliferated rapidly in response to endotoxin. High-proliferative-potential colony-forming cells (HPP-CFC) were tested as a candidate for this precursor population. The results of the determination of the kinetics for these cells showed that the HPP-CFC exist largely in a G_0 state, exiting at an average rate of once every four days. The slow turnover time for these cells and their response to endotoxin challenge are consistent with a close relationship between the HPP-CFC and the G_0 pool of cells that is the direct precursor of the GM-CFC.

Key words: HPP-CFC — GM-CFC — BrdUrd — Kinetics

Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

Research was conducted according to the principles enunciated in the "Guide to the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

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Upon their isolation from normal murine bone marrow, myeloid/monocytic cells that form colonies in agar (GM-CFC) represent a heterogeneous population [1-3]. These heterogeneities can be detected through physical properties of the cells [1, 2], differences in membrane receptors [4-6], or the relative proportion of cells in the various phases of the cell cycle [7, 10, 14]. The separation of GM-CFC subpopulations based on buoyant density [2, 8], sedimentation velocity [1], S-phase content [7, 10], or their toxic response to specific drugs [7, 9] has led several workers to conclude that the basis for the heterogeneity in GM-CFC may be either the proliferative future of the cell or the state of differentiation [11-13]. As an example, colony size, which is presumably related to the proliferative future of the colony-forming cell, has been recently shown to identify a relatively distinct population of the GM-CFC [9, 13]. Using this discriminator, Bradley and co-workers identified a minor GM-CFC subpopulation, the "high-proliferative-potential colony-forming unit" (HPP-CFC), which appears to be a candidate precursor for the majority of the GM-CFC [9, 13]. These cells, optimally expressed as colonies only in the presence of human serum and spleen-conditioned medium, are largely resistant to S-phase-specific cytotoxic agents [9, 13, 15].

In recent work, while measuring the cell-cycle kinetics of the GM-CFC under conditions that limited the expression of HPP-CFC, we observed a subpopulation of GM-CFC that appeared to have recently come from a pool of noncycling cells [14]. Since this transition is likely to be important to the understanding of the relationship between the HPP-CFC and the general GM-CFC population, we have examined in detail the BrdCyd-labeling kinetics of the cell populations involved.

To examine these kinetics, the changes in sensitivity of GM-CFC and HPP-CFC to 313-nm light were measured over an extended period of BrdCyd infusion. The kinetics for the development of 313-nm light sensitivity for initially ultraviolet (UV) resistant GM-CFC are consistent with these cells having recently entered the cell cycle from a pool of G_0 cells. In addition, the turnover time for this pool of

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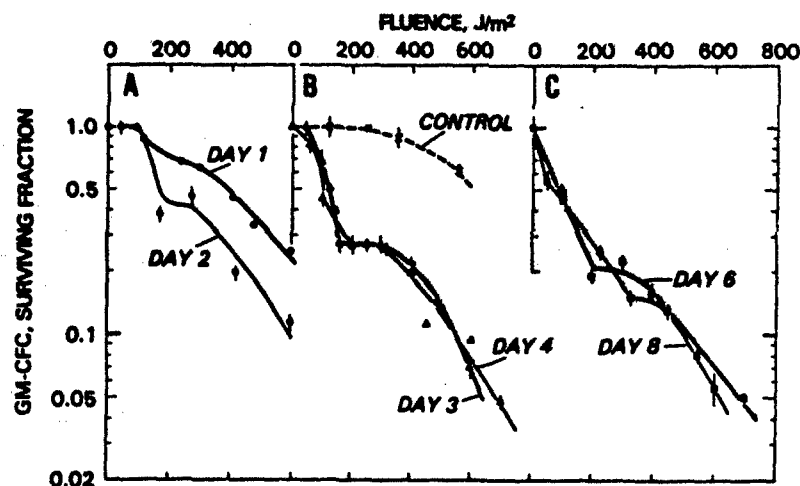


Fig. 1. GM-CFC survival after BrdCyd/313-nm-light treatment. After BrdCyd infusion for the period shown, cell suspensions of GM-CFC were exposed to the fluence of 313-nm light indicated. Control cultures were derived from animals not receiving BrdCyd. Error bars representing ± 1 SE are shown when they are larger than the symbol. Data represent the mean of triplicate samples.

G_0 cells and their response to endotoxin challenge are consistent with a close relationship between the cells within this G_0 pool and the HPP-CFC [9, 13].

Material and methods

Mice. B6D2F, female mice, 12–16 weeks of age (Jackson Laboratory, Bar Harbor, ME), were used throughout. Control and experimental mice, randomized with respect to age, were maintained on a 6:00 AM to 6:00 PM light-dark cycle. Wayne Lab-Blox and hyperchlorinated water were available ad libitum. Prior to treatment, mice were acclimated to laboratory conditions for two weeks. During this time, the mice were examined and found to be free from lesions of murine pneumonia complex and of oropharyngeal *Pseudomonas* sp.

BrdCyd labeling. BrdCyd labeling has been described elsewhere [14, 21]. Briefly, BrdCyd labeling was accomplished with indwelling osmotic minipumps filled with 150 mM BrdCyd (Calbiochem-Behring, San Diego, CA) in sterile, pyrogen-free water. Minipumps (Alza Corporation, Palo Alto, CA) were implanted subcutaneously on the dorsal surface during chloralhydrate anesthesia.

313-nm-light irradiation. Murine bone marrow cells, suspended at 5.0×10^6 cell/ml in Dulbecco phosphate-buffered saline, were irradiated with monochromatic 313-nm light (10-nm band width) at a flux of $15.0 \text{ J/m}^2/\text{s}^{-1}$. The irradiation source consisted of a 1 kW Hg-Xe lamp (Oriol Corporation, Stamford, CT) filtered through 80 mm of $1.0 \times 10^{-4} \text{ M}$ dAd and focused onto a monochromator (model 7024, Oriol Corporation). The flux was determined with a model 8334A thermopile and microammeter (Hewlett-Packard, Palo Alto, CA).

GM-CFC assay. On each day of the infusion period, cell suspensions were prepared of pooled axially derived bone marrow from the femurs of three mice. GM-CFC [16] were assayed by the double-layer agar technique. The agar medium was plated into 60-mm plastic Petri dishes as follows: bottom layer, 2 ml of 1.0% agar (Bactoagar, Difco, Detroit, MI), 2 ml double-strength medium, 100 μl pregnant-mouse-uterus extract [17], 150 μl mouse L-cell-conditioned medium [18]; top layer, 1 ml of 0.66% agar and 1 ml double-strength medium. Double-strength medium

consisted of Connaught Medical Research Laboratory (CMRL, Toronto, Canada) 1066 medium containing 10% (vol/vol) fetal calf serum, 5% (vol/vol) horse serum, 5% (wt/vol) trypticase soy broth, 20 g/ml L-asparagine, and antibiotics. After 10 days of incubation at 37°C in 5% humidified CO_2 in air, GM-CFC were scored as colonies of more than 50 cells. For HPP-CFC, the same double-layer agar technique was employed as by Metcalf and Johnson [19], but rat spleen cell-conditioned medium (CM) and PMUE were used as colony stimulants. Single-cell suspensions of rat spleen were prepared at a concentration of 16×10^6 cells/ml of medium. The pokeweed-mitogen-spleen-cell CM was prepared as described by Metcalf and Johnson [19]. Rat spleen CM and PMUE were each used at 6% (vol/vol). The culture medium contained 25% heat-inactivated, pooled human serum. The marrow cells were plated at 5×10^4 cells/dish. An eyepiece graticule was used to assess colony size. All colonies greater than 2 mm in diameter were considered to be derived from HPP-CFCs. Experiments were routinely performed in triplicate with the mean and standard errors reported on the Figures. The data in Figure 5 represent duplicate experiments.

Results

The time dependence of the BrdCyd labeling of GM-CFC was measured for infusion periods of 1–8 days. For these measurements, cell suspensions were exposed to graded fluences of 313-nm light, after which the surviving fraction of GM-CFC was determined. For each of the resulting survival curves, two components of UV sensitivity were detectable. As can be seen in Figure 1, there are two components of cell survival, the more resistant observed for fluences greater than 200 J/m^2 . The proportion of cells more resistant to 313-nm light decreased from approximately 70% after one day of infusion to approximately 15% after eight days.

This decrease in the size of the resistant populations was further analyzed by plotting in Figure 2 the proportion of 313-nm-light-resistant cells versus time. GM-CFC survival values at a fluence of ~ 300

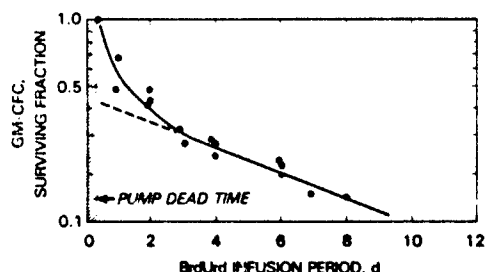


Fig. 2. Decrease in the proportion of GM-CFC resistant to 313-nm light (see text). The fraction of resistant GM-CFC is estimated from the fraction of GM-CFC surviving a fluence of 300 J/m^2 . Pump dead time represents the time from the implantation of minipumps to the time at which BrdCyd is present in the peripheral blood.

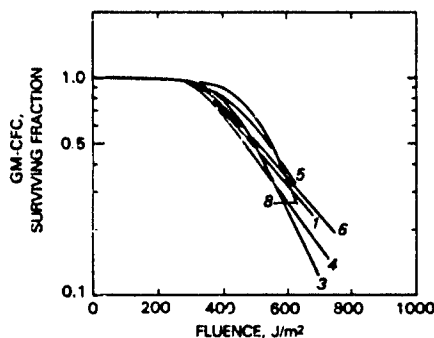


Fig. 3. Survival of the UV-resistant component of the GM-CFC population. The data presented in Figure 1 were normalized to unity at a fluence value of 300 J/m^2 . The resultant survival curves are indicated by a number corresponding to the number of days of BrdCyd infusion.

J/m^2 were used to estimate the proportion of colony-forming cells in the resistant cell population. From the resulting curve, one notes that initially none of the GM-CFC are sensitized to 313-nm light. Over the initial two days of BrdCyd labeling, however, approximately 55% of the GM-CFC rapidly became sensitive to 313-nm light while the remaining 45% were sensitized considerably more slowly. The slowly labeled component demonstrated a half-time for labeling of 5.5 days. In addition to the decrease in the 313-nm-light-resistant cell population, two other characteristics of the survival data in Figure 1 are of interest. First, for fluence values less than 100 J/m^2 , a shoulder appears on the day-1 and day-2 survival curves. This shoulder was reduced to less than 50 J/m^2 for the survival curve determined at day 4 and eliminated by day 8. Secondly, the fluence value of the inflection in the survival curve appears not to be a function of the infusion period. This inflection identifies the 313-nm-light-resistant cell population described

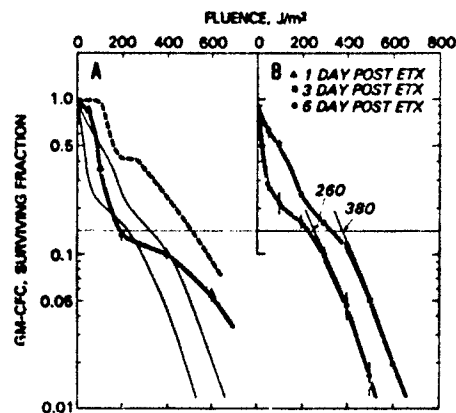


Fig. 4. Effect of endotoxin (ETX) on GM-CFC survival after BrdCyd/313-nm-light treatment. Endotoxin was administered (see Materials and methods) 24 h after the implantation of minipumps. For comparison, the survival data (shown by the dashed line in A) represent normal GM-CFC after 48 h of BrdCyd infusion.

above. By normalizing to unity the survival values at 300 J/m^2 (i.e., fluence value of the secondary shoulder), one sees in Figure 3 that no significant change in the fluence value of this shoulder region occurs during the eight days of BrdCyd infusion. The significance of these shoulder regions will be addressed later in detail.

To determine whether either GM-CFCG population could respond to a proliferative stimulus, endotoxin was administered to a group of mice 24 h after the implantation of BrdCyd-containing minipumps. The intraperitoneal administration of $2.5 \mu\text{g}/\text{mouse}$ of *Salmonella typhosa* endotoxin produced changes in two of the survival curve characteristics described above. The data in Figure 4A show that the fraction of 313-nm-light-resistant cells dropped to approximately 15% 24 h after the endotoxin challenge and was essentially unchanged over the subsequent five days of infusion. In addition, the sensitivity of this population to 313-nm light appeared to increase with time; that is, a comparison of the width of the shoulder at the 15% survival level revealed a measurable decrease as the period of infusion increased. This shift in the shoulder value contrasts directly with the data for the nonchallenged hosts, viz., Figure 3.

Both the G_0 nature and the responsiveness to endotoxin of the GM-CFC precursor cell are characteristics associated with the HPP-CFC [13, 20]. It was decided, therefore, to measure the kinetics of BrdCyd labeling of this cell type. The HPP-CFC was cultured and identified with the techniques detailed in Materials and methods. Additional confirmation of the HPP-CFC was provided through the small

Table 1. Decrease in femoral content^a of HPP-CFC and GM-CFC 2 h after injection with hydroxyurea (Hu)^b

| | HPP-CFC | | | GM-CFC | | |
|----|--------------------|---------|------------|---------------------|---------|------------|
| | Pre-Hu | Post-Hu | % Decrease | Pre-Hu | Post-Hu | % Decrease |
| 1) | 6471 | 5805 | 10.3 | 18,531 | 13,496 | 27.2 |
| 2) | 8308 | 8088 | 2.6 | 20,689 | 11,844 | 42.8 |
| 3) | 5420 | 4607 | 15.0 | 18,432 | 11,300 | 38.4 |
| 4) | 7175 | 6583 | 8.3 | 36,600 | 17,460 | 52.3 |
| | Mean 9.1 ± 2.6 | | | Mean 40.2 ± 5.2 | | |

^a Mean values from four individual replicate experiments are shown. Four mice were used per replicate. Femoral contents were pooled. HPP-CFC and GM-CFC assays were performed on the same marrow samples.

^b Hu, 900 mg/kg, was injected intraperitoneally.

fraction of these cells sensitive to hydroxyurea, a characteristic of the HPP-CFC [13]. The data, shown in Table 1, relate this property and agree well with the published difference between GM-CFC and HPP-CFC [13]. If, indeed, the HPP-CFC form this pool of cells that are precursors of the GM-CFC, then they should survive the BrdCyd/313-nm-light treatment in a predictable manner. They should be non-cycling. Therefore, there should be no S-phase population to give rise to a shoulder at 200–300 J/m². Further, a plateau in survival should be present for each infusion period. This plateau value should decrease at the rate shown in Figure 2. Survival curves illustrating this ideal behavior and actual HPP-CFC survival data are presented in Figure 5. In Figure 5A, survival curves with no shoulder and a minimum or plateau are shown for increasing periods of BrdCyd labeling. When these plateau values are plotted against their associated infusion periods, the resulting simple exponential curve mimics the curves shown in Figure 2. The survival curves for the HPP-CFC shown in Figure 5B were derived from the mean values of duplicate experiments. Also shown for comparison are the survival curves for the GM-CFC population measured from the same pool of cells as one of the HPP-CFC determinations.

Discussion

The present work reports the cell survival response after BrdCyd/313-nm-light treatment of GM-CFC derived from murine bone marrow. BrdCyd infusion was used to effect BrdUrd labeling via the *in vivo* deamination of BrdCyd [21, 22]. The kinetics for the BrdCyd labeling have been determined by measuring the time rate of change in sensitivity to 313-nm light of the GM-CFC population.

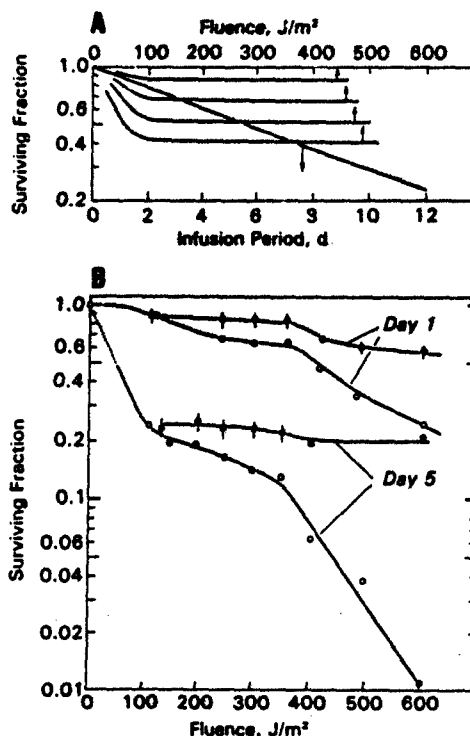


Fig. 5. Survival of HPP-CFC after BrdCyd/313-nm-light treatment. (A) Theoretical survival curves for the precursor of the GM-CFC. Minimum (plateau) values in survival were determined from the data in Figure 2. (B) Actual survival data for the HPP-CFC (●) and GM-CFC (○) for the infusion periods shown. HPP-CFC data points represent the mean value \pm 1 SE for duplicate experiments ($n = 6$). GM-CFC data points are mean values from duplicate samples.

From the data in Figures 1–4, four characteristics of the BrdCyd labeling can be defined. First, as expected, the fraction of the GM-CFC initially resistant to 313-nm light is near unity. During the BrdCyd-labeling period, however, this UV-resistant fraction decreases. The pattern of this decrease in survival discloses two subpopulations with significantly differing labeling kinetics. The more rapidly labeled GM-CFC subpopulation comprises approximately 55% of the total GM-CFC and the more slowly labeled cells comprise the remaining 45%. Secondly, the sensitivity to 313-nm light of the UV-resistant subpopulation does not change with time. However, a steady-state decrease is seen in the proportion of cells resistant to 313-nm light. This decrease is approximately exponential with a half-time of 5.5 days. Third, the proportion of UV-resistant cells decreases markedly in the first 24 h for mice injected with endotoxin. Lastly, in contrast with the normal steady-state response, mice injected with endotoxin exhibit an increase in the sensitivity of the UV-resistant GM-CFC component; this effect

is observed by a decreasing shoulder value with increasing periods of BrdCyd infusion.

To interpret these results, it is first necessary to consider the possible routes for the *in vivo* BrdCyd labeling of any population of proliferating cells. From data obtained *in vitro* [22], one knows to expect two components of BrdCyd labeling, both with rapid kinetics (i.e., half-times of less than one cell cycle). These two components arise through (a) the incorporation by cells in the S-phase of a sufficient amount of BrdUrd to be conditionally lethal upon exposure to UV light, and (b) the progress into S-phase of cells initially not synthesizing DNA. However, *in vivo* a third route (*viz.*, the differentiation into the compartment being assayed of cells labeled in the precursor cell compartment) represents an alternate pathway for the accumulation of labeled cells. This pathway for the accumulation of BrdUrd-labeled cells appears to be *apropos* for the UV-resistant GM-CFC. Unlike the data for experiments performed *in vitro*, which show near-maximum sensitivity to 313-nm light after one or two generation times, the data presented here show a population of GM-CFC that is fully sensitized only after some eight or more days, a period much longer than the GM-CFC generation time that would be estimated from the results of suicide experiments, *viz.*, approximately 12 h [23]. Moreover, the UV sensitivity of this population does not change with time, yet it has been shown earlier that these cells have an S-phase fraction of approximately 50% [14]. Thus, these cells are not themselves postmitotic. Since these GM-CFC are cycling in the steady state but are "seeing" the BrdCyd label for the first time, it is likely that they have recently entered the assayable GM-CFC compartment from a noncycling, or G_0 , population not assayable under the same conditions.

If the GM-CFC that are initially resistant to 313-nm light are supplied to the GM-CFC compartment directly from a pool of proliferating cells, their UV sensitivity would presumably increase with time. Thus, in time, the fluence value for the shoulder region of the survival curve would decrease and the slope of the survival curve would increase. This does not happen. If the precursor population were composed largely of a G_0 fraction, however, those GM-CFC derived from the G_0 would possess just such properties as described for the slowly labeled 313-nm-light-resistant cells; that is, the shoulder value would not change with time, but the proportion of resistant cells would. Furthermore, the rate of the decrease with time of the proportion of these cells resistant to 313-nm light implies that the precursor population is itself supplied at a rate consistent with

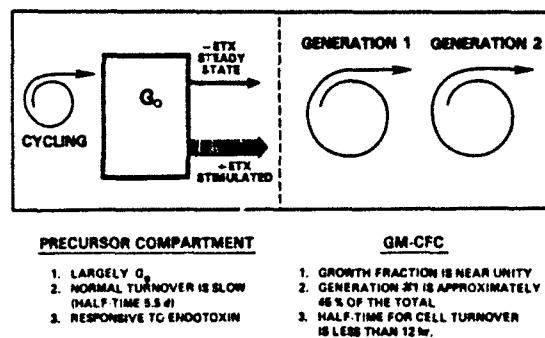


Fig. 6. Kinetics model for GM-CFC production (see text for explanation).

a 5.5-day half-time for the turnover of the G_0 cell pool. Furthermore, the G_0 nature of the precursor of the GM-CFC requires the precursor population to be either fully labeled or completely unlabeled with BrdCyd. This point will be important later.

The GM-CFC precursor pool was also manipulated through its response to a stimulus to proliferate. After the administration of endotoxin, this G_0 pool was exhausted. Whether this occurred through cell proliferation or cell mobilization from the marrow cannot be determined from these data alone. The effect of each would be the same; *viz.*, each process would remove unlabeled GM-CFC from the population responsible for the time-dependent increase in sensitivity to 313-nm light. Interestingly, after the administration of endotoxin and the subsequent depletion of the G_0 pool, a time-dependent increase in sensitivity to 313-nm light occurred (Fig. 4). This effect was most likely due to a recent episode of DNA synthesis for the cells filling this GM-CFC precursor pool. This increased sensitization to UV light may also occur in the absence of an endotoxin challenge and simply have been obscured in the unchallenged animal by the presence of the large fraction of precursor cells that are normally in a G_0 -phase and thus are not labeled by the BrdCyd. Therefore, this increase in sensitivity to UV light apparently induced by the endotoxin may not be dependent upon the endotoxin challenge but may only have been rendered detectable by it.

A model that is consistent with these findings is presented in Figure 6. Although the model is quite general, it is constrained at several points by the present data. For example, the model has been limited to two generations of GM-CFC by the high percentage of GM-CFC that appear to be in the first generation of labeling (i.e., 45% shown in Fig. 2). Furthermore, the G_0 pool is shown as a precursor pool and not a GM-CFC reservoir. This is due prin-

cipally to the high S-phase content of both GM-CFC moieties [14]. The previously reported lower buoyant density of the GM-CFC that are being labeled for the first time is a further indication that first-generation GM-CFC are progeny of the physiologically distinct precursor pool [2, 3, 14]. The final constraint upon the model is that the GM-CFC compartment is being fed by a separate cell type and not by self-renewal. This property has been suggested by others on the basis of the inability to propagate GM-CFC in vitro [24, 25]. Here, the evidence against self-renewal is that first-generation GM-CFC are labeled much more slowly than the rate at which the GM-CFC are cycling. Self-renewal in the GM-CFC compartment thus appears to be limited to, at most, two generations.

The cell kinetics of one candidate for the GM-CFC precursor, the HPP-CFC [9, 13], were measured, and the data are presented in Figure 5. This cell type has previously been shown to be both slowly proliferating, if at all, and responsive to endotoxin challenge [20]. Both of these are properties consistent with the data presented here for the GM-CFC precursor. Further, from the present data, both qualitative and quantitative predictions can be made concerning the survival after 313-nm-light irradiation of the HPP-CFC. If the HPP-CFC constitute the pool of cells in G_0 that feed into the GM-CFC, the HPP-CFC should be composed initially of cells largely not capable of being labeled during the first 24 h of BrdCyd infusion. Secondly, since a G_0 population by definition has no cells in the S phase, there should be no cell population to produce a shoulder on the UV survival curve [26, 27]. Therefore, the survival curves for the HPP-CFC should possess two components: one fully sensitive and the other fully resistant to the BrdCyd/313-nm-light treatment. Further, the proportion of fully resistant HPP-CFC should decrease in a manner quantitatively similar to the decrease in the 313-nm-light-resistant GM-CFC shown in Figure 2.

The data in Figure 5 show that the HPP-CFC labeled for one day are largely not sensitized to 313-nm light. However, a minor population of cells gives rise to a shoulder on the survival curve. For day 5, no such shouldered population was observed. Quantitatively, the population of cells resistant to 313-nm light is below the values predicted from the data presented in Figure 2. For both day 1 and day 5 of the BrdCyd infusion, the nonsensitized fraction is approximately 30% below the predicted values. Since 30% is also the approximate proportion of cells giving rise to the shoulder on the survival curve for day 1 of the infusion, this population may simply

be a GM-CFC contaminant of the HPP-CFC assay. In fact, such a contaminant has been reported by others when human serum and mouse spleen-conditioned medium are used to induce the expression of HPP-CFC [13]. Using the plateau survival values from Figure 5, the half-time for turnover of the HPP-CFC is approximately four days. These quantitative differences notwithstanding, the qualitative pattern of survival of the HPP-CFC after BrdCyd/313-nm-light treatment is consistent with the predictions made for the GM-CFC precursor. Although these data support the notion that the HPP-CFC give rise to the GM-CFC, there is no indication from these assays of the nature of the proliferative cell population that supplies the HPP-CFC. The present data suggest that, if the HPP-CFC is the precursor of the GM-CFC, then the HPP-CFC must be supplied from a proliferative cell compartment at a rate that, in the steady state, is consistent with a 5.5-day half-time for cell turnover.

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